(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 March 2002 (14.03.2002)

PCT

(10) International Publication Number
WO 02/19832 A1

(51) International Patent Classification[7]: A23C 9/00, A61K 35/20

(21) International Application Number: PCT/NZ01/00186

(22) International Filing Date:
10 September 2001 (10.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
506827 8 September 2000 (08.09.2000) NZ

(71) Applicant *(for all designated States except US)*: **NEW ZEALAND DAIRY BOARD** [NZ/NZ]; Pastoral House, 25 The Terrace, Wellington (NZ).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CRAWFORD, Robert, Andrew** [NZ/NZ]; New Zealand Dairy Research Institute, Fitzherbert West, Palmerston North (NZ). **BOLAND, Michael, John** [NZ/NZ]; New Zealand Dairy Research Institute, Fitzherbert West, Palmerston North (NZ). **NORRIS, Carmen, Sarah** [NZ/NZ]; New Zealand Dairy Research Institute, Fitzherbert West, Palmerston North (NZ). **HILL, Jeremy, Paul** [GB/NZ]; New Zealand Dairy Research Institute, Fitzherbert West, Palmerston North (NZ). **FENWICK, Robin, Milson** [NZ/NZ]; 32 Rothsay Road, Ngaio, Wellington (NZ).

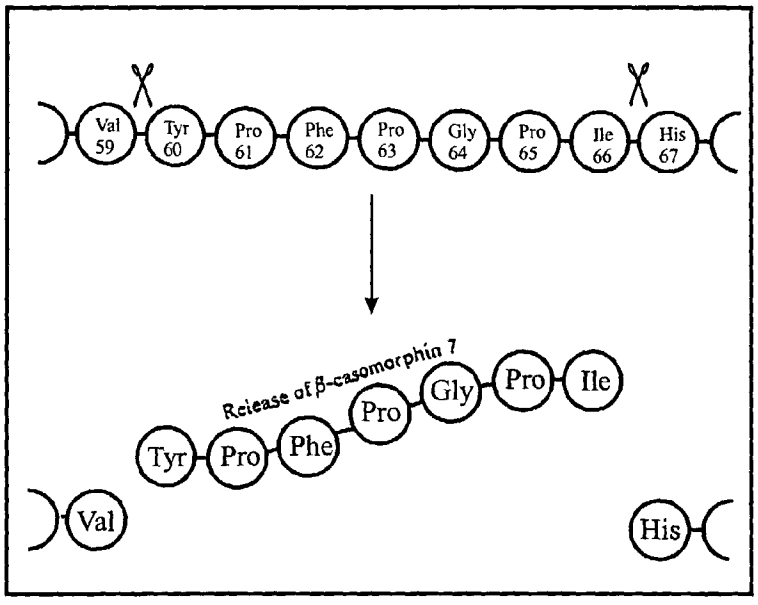
(74) Agent: **A J PARK**; Calhoun, Douglas, C., 6th Floor, Huddart Parker Building, Post Office Square, P.O. Box 949, Wellington 6015 (NZ).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,

[Continued on next page]

(54) Title: MILK CONTAINING β-CASEIN WITH PROLINE AT POSITION 67 DOES NOT AGGRAVATE NEUROLOGICAL DISORDERS



(57) Abstract: The invention is based on the discovery that the consumption of milk which contains a β-casein variant which has histidine or any other amino acid not proline at position 67, may on digestion cause the release of an opioid which may induce or aggravate a neurological/mental disorder such as autism or Asperger's syndrome. The invention is supplying milk or milk products that contain β-casein with proline at position 67 to susceptible individuals.

WO 02/19832 A1

CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:

— *with international search report*

Milk containing β-casein with proline at position 67 does not aggravate neurological disorders

TECHNICAL FIELD

This invention relates to the selection of milk which contains certain β-casein variants and the use of such milk and/or other food products containing components of this milk to provide nutrition in the diet of a susceptible individual without inducing or aggravating a neurological/mental disorder such as autism spectral disorders in the individual.

BACKGROUND ART

Milk is designed to provide total nutrition to newborn mammal and for this reason contains a wider range of nutrients than most other foods. Milk is nutrient dense in certain essential components such as calcium, amino acids and other macro- and micro-nutrients important for healthy development, particularly of infants and children (Hamraeus, 1992, Flynn and Cashman, 1997). Milk components are an important ingredient in a number of formulated or processed foods and are often used to improve the nutritional value of such foods. Milk and milk product consumption are thus considered to be an important part of a healthy balanced diet.

It has been proposed that some neurological/mental disorders could be the consequence of the action of opioid peptides of exogenous origin affecting normal function of the central nervous system (CNS) (Panksepp, 1979, Shattock *et al.*, 1990, Reichelt *et al.*, 1981, Shattock and Lowdon, 1991, Williams *et al.*, 1991, Brudnak, 2001). The presence of intense opioid activity can result in the CNS being disrupted to the point where perception, cognition, emotions, mood and behaviour become affected. In the case of schizophrenia and autism, it has been suggested that biologically active peptides derived from the diet may influence these diseases (Reichelt *et al.*, 1981, Shattock and Lowdon, 1991). Some authors have recommended that patients suffering from disorders such as autism or schizophrenia should exclude foods capable of producing opioids, such as dairy or cereal products, from their diets, and there have been anecdotal reports of some patients responding favourably to such diets (Knivsberg *et al.* 1990). Patients suffering from disorders such as autism are thought to have a digestive tract that is functioning suboptimally and/or is leaky resulting in incomplete digestion of casein and

glutin to produce casomorphins and gluteomorphins that cross the lumen of the gut into the circulation (Brudnak, 2001).

β-Casein contains a sequence of amino acids that correspond to, and give rise to the bioactive peptides called β-casomorphins (BCMs). These peptides are known to have opioid activity (see for example a review by Miesel, 1997). The β-casomorphins are a family of peptides made up from or including the sequence TYR-PRO-PHE-PRO-GLY-PRO-ILE. This sequence corresponds to residues 60 to 66 of the β-casein protein. The example shown is known as β-casomorphin-7 (BCM-7) because it is 7 residues long. Other forms of β-casomorphin are similarly numbered, always starting from the TYR at position 60 of β-casein. A further peptide of relevance is the peptide that contains the BCM-7 sequence, plus an additional VAL on the N-terminal (VAL-BCM-7). This corresponds to the sequence from residues 59 to 66 in β-casein. The peptides of particular relevance to this document are BCM-7, BCM-6 and VAL-BCM-7. BCM-7 and VAL-BCM-7 have been observed in urine samples of autistic patients. See EP969015 beginning at paragraph 0056 and in particular paragraph 0090 and Figure 8.

Some individuals are predisposed to physiological responses to dietary challenge. An individual may for example, have a compromised gut wall that allows transit of food fragments, or may not secrete necessary enzymes to fully degrade food proteins, or some other (temporary or permanent) biological/physiological process may apply. The provision of peptides that originate from a food protein could then trigger a response in that individual which is not observed in other individuals. Removal of the source of such food components from the diet of susceptible individuals would be a useful contribution to their health.

Recently it was shown that there was a strong positive relationship between the consumption of milk which contained the A1 or B variants of the milk protein β-casein and insulin-dependent *diabetes mellitus* (Elliott *et al.*, 1999). Elliott *et al.* (1999) found that only those β-casein variants which had a histidine at position 67 of their amino acid sequences were correlated with the disease. Hartwig *et al.* (1997) have shown that *in-vitro* BCM-7 could only be released by digestive enzymes from those β-casein variants with a histidine at position 67 in the primary sequence of this protein (β-casein A1, B and C). The peptide was not released from β-casein variants with a proline in this position (β-casein A2 and A3).

For the purpose of this specification, any β-casein variant which has a histidine at position 67 of its amino acid sequence (or any other amino acid other than a proline at position 67) will be referred to as a "histidine" variant. Any β-casein variant which has a proline at position 67 of its amino acid sequence will be referred to as a "proline" variant. Table 1 shows the differences of β-casein variants from A to F by position of the amino acid residue in the polypeptide chain, with the A2 variant taken as the base structure.

TABLE 1

Variant	**18**	**35**	**36**	**37**	**67**	**106**	**122**	**152**
A1					His			
A2	**Ser-P**	**Ser-P**	**Glu**	**Glu**	**Pro**	**His**	**Ser**	**Pro**
A3					Pro	Gln		
B					His		Arg	
C		Ser		Lys	His			
D	Lys				Pro			
E			Lys		Pro			
F					His			Leu

In light of this it can be concluded that it would be beneficial to exclude from the diet of patients susceptible to mental disorders milk which contains histidine variants while at the same time providing such patients with milk containing proline variants so as to provide the nutritional benefits of milk without adverse side effects.

It is an object of this invention to go some way towards achieving this desideratum or at least to offer the public a useful choice.

DISCLOSURE OF THE INVENTION

Accordingly, the invention may be said broadly to consist in a method of providing nutrition to a susceptible individual while avoiding the induction or aggravation of a neurological/mental disorder, which comprises supplying to said individual milk which does not contain any histidine variant.

Alternatively, said method comprises supplying to a susceptible individual a milk product incorporating only milk which does not contain a histidine variant.

Preferably said mental disorder is autism spectral disorder.

Preferably said autism spectral disorder is autism, pervasive developmental disorder or Asperger's syndrome.

Preferably said milk product includes a non-dairy food ingredient which does not contain a histidine variant.

Preferably said milk product includes a nutraceutical.

Preferably said milk is bovine milk.

Alternatively said milk is derived from mammals other than bovine.

Preferably said alternative milk is derived from goats or sheep.

In another embodiment the invention may be said broadly to consist in the use of milk having no histidine variant in the manufacture of a milk product for providing nutrition to a susceptible individual while avoiding induction or aggravation of a neurological/mental disorder in said individual.

Alternatively the invention consists in the use of a milk product so manufactured in the manufacture of a nutrient composition incorporating only nutrients which contain no histidine variant.

Preferably said neurological/mental disorder is autism spectral disorder.

Preferably said autism spectral disorder is autism, pervasive developmental disorder or Asperger's syndrome.

Alternatively said nutrient composition includes nutraceuticals.

Preferably said milk is bovine milk.

Alternatively said milk is derived from mammals other than bovine.

Preferably said alternative milk is derived from goats or sheep.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention may also be more fully understood by having reference to the accompanying drawings wherein:

Figure 1 shows a representation of β-casein variants having histidine at position 67 of its amino acid sequence which permits the formation of betacasomorphin 7. This illustrates the release of BCM-7 from the variants A1, B, C and F all of which have histidine at position 67 of their amino acid sequences.

Figure 2 illustrates that when there is a proline at the 67 position which occurs in variants A2, A3, D and E the bond between the amino acids at position 66 and 67 does not cleave and BCM-7 is not released.

Figure 3 is a plot of deaths due to mental disorders per 100,000 population on the Y axis against consumption of total milk protein in grams per person per day on the X axis.

Figure 4 is a plot of deaths due to mental disorders per 100,000 population on the Y axis against A1 β-casein consumption in grams per person per day on the X axis.

Figure 5 is a plot of deaths due to mental disorders per 100,000 population on the Y axis against consumption of A1 and B variants of β-casein in grams per person per day on the X axis.

Figure 6 is a plot of deaths due to mental disorders per 100,000 population on the Y axis against consumption of A2 and A3 variants of β-casein in grams per person per day on the X axis.

Figure 7 shows a time course for the rate of formation of β-CM-7 in an *in vitro* hydrolysis of A1/A2 casein

Figure 8a. Total ion current trace for in vitro hydrolysis of A1 casein showing Val-BCM-7 eluting at approximately 54 minutes.

Figure 8b. Extracted ion chromatogram of the peak at 54 mins from Figure 8a.

Figure 9a. total ion current trace for in vitro hydrolysis of A2 casein showing Val-BCM-7 eluting at approximately 54 minutes.

Figure 9b. Extracted ion chromatogram of the peak at 54 mins from Figure 9a.

Figure 10 shows the level of BCM-6 in the urine of an autistic individual following the consumption of histidine variant containing milk and proline variant containing milk.

Figure 11 shows the level of BCM-6 in the urine of an autistic individual following the consumption of histidine variant containing milk.

Figure 12 shows the level of BCM-6 in the urine of an autistic individual following the consumption of proline variant containing milk (note the expanded X-axis).

MODES OF CARRYING OUT THE INVENTION

Relationship between Neurological/Mental Health and β-Casein Variant Consumption

The average consumption of milk protein and β-casein A1 and B (gm/day) was calculated using the method from Elliott R B, et al (1999). The contents of this are incorporated by reference.

The β-casein variant consumption was calculated using the following equation:

$$\mathbf{C = \Sigma(f \times B) \times P \times Y}$$

Where

C = consumption of β-casein of the particular variant(s)

f = frequency of the particular β-casein allele(s) in the breed in the national dairy herd

B = the proportion of the breed in the national dairy herd

P = the mean daily intake of dairy protein (from FAOSTAT database)

Y = the fraction of β-casein as a proportion of all protein in milk

The countries and regions selected were Australia, Canada, Denmark, Finland, Germany, Iceland, New Zealand, Norway, Sweden and San Diego (USA). In the case of Iceland the amount of β-casein variants in milk was determined by chemical analysis. All the countries selected had low imports of dairy products from other countries.

<u>β-casein variants in Icelandic milk were calculated as follows:</u>

Milk from Iceland was freeze dried and shipped to New Zealand for analysis. The sample was reconstituted in water (130 mg/ml) and mixed for three hours. Exactly 0.5 ml of sample was added to 2.5 ml of sample buffer at pH 8.6 (6 M urea, 42 mM MOPS, 0.05% methyl cellulose and 167 mM Tris). Mercaptoethanol (30 mL) was added to the sample which was incubated for one hour at room temperature prior to filtering. The sample was then subjected to capillary electrophoresis on an Applied Biosystems 270A-HT capillary electrophoresis system using a Supleco hydrophobically coated capillary. Samples were eluted from the capillary using a running buffer (6 M urea, 20 mM sodium citrate, 032 mM citric acid and 0.05% methyl cellulose at pH 3.0).

<u>Death rates due to mental disorders were determined as follows:</u>

Death rate attributable to mental disorders were sourced from World Health Organisation's web site (www.who.int/whosis/). This web site has recent data (1994-1998) from a wide range of data. The 1995 data were used where possible.

From Figure 3 it can be seen that the consumption of total milk protein in Iceland is higher than that in Finland, Sweden and Norway. However, Iceland has a very low level of mental disorders while Norway, Sweden and Finland rank much above the middle countries.

However, when the consumption of the β-casein variant is calculated (Figures 4 and 5), the consumption of the A1 and the A1 + B (histidine) variants in Iceland is much lower than that in Norway, Sweden and Finland. This is consistent with a correlation between mental disorders and the consumption of histidine variants. Figure 6 shows a high level of consumption of proline variants in Iceland, and a low level of mental disorders.

Statistical analysis

The data presented in Figures 3 to 6 were analysed using Pearson's correlations. Pearson's correlations are a measure of the degree of linear association between two variables. A correlation of +/- 1 means the data lies on a straight (or linear) line when plotted against each other. A correlation of 0 means there is no relationship between the two variables. T-tests were used to test the strength of the correlation, taking into account the number of datapoints in the data set. These generate a p-value. The p-value is the probability of obtaining as high a correlation as was obtained by chance effects alone. A p-value < 0.05 was accepted as evidence that the correlation was statistically significant. The linear regression coefficients (r) of the lines in the plots were determined. Results are summarised in Tables 2 and 3. Table 3 excludes the results from Iceland.

TABLE 2

Correlations (Pearson) - Including Iceland

Type	All Deaths due to Mental Disease	Male Deaths due to Mental Disease	Female Deaths due to Mental Disease
A1 (his variant)	0.795[1] 0.006[2]	0.731 0.016	0.801 0.005
A1 + B (his variants)	0.787 0.007	0.744 0.014	0.783 0.007
A2 + A3 (pro variants)	-0.219 0.544	-0.379 0.280	-0.137 0.706
Milk Protein	0.124 0.720		

[1] r- value [2]p- value

TABLE 3

Correlations (Pearson) - Without Iceland

Type	All Deaths due to Mental Disease	Male Deaths due to Mental Disease	Female Deaths due to Mental Disease
A1	0.855[1]	0.863	0.832
(his variant)	0.003[2]	0.003	0.005
A1 + B	0.758	0.731	0.751
(his variants)	0.018	0.025	0.020
A2 + A3	0.584	0.584	0.570
(pro variants)	0.099	0.099	0.109

[1] r- value [2]p- value

***In-vitro* Hydrolysis of β-Casein Variants**

Two batches of mineral casein were made according to standard procedures. Each batch of casein had been made from milk collected only from cows with β-CN phenotypes A1/A1 or A2/A2.

A1 and A2 casein were enzymatically hydrolysed according to an *in vitro* experiment to mimic the digestion process of the stomach and gut based on methodology developed by Petrilli et al. (1984), Svedberg et al. (1985) and Pihlanto-Leppälä et al. (1994).

All enzymes were purchased from Sigma (St. Louis, Missouri, USA): pepsin (Catalogue Number (C/N) P-6887), trypsin (C/N T-0134), chymotrypsin (C/N C-3142), elastase (C/N E-1250), carboxypeptidase A (C/N C-9268), carboxypeptidase B (C/N C-9584) and peptidase (C/N P-7500). Hydrolysis was carried out according to the protocol in Table 4.

TABLE 4

In vitro hydrolysis procedure

Time (min)	Enzyme added	Enzyme to substrate ratio	Temperature (°C)	pH
0	Pepsin	1%	50	2.0
60	Trypsin	1%	50	8
120	Chymotrypsin	1%	50	8
180	Elastase	0.96%	50	8
240	Carboxypeptidase A	0.87%	50	8
300	Carboxypeptidase B	0.2%	50	8
360	Peptidase	1%	50	8
420	-	-	50	8

Samples were taken before enzyme addition at 60, 120, 180, 240, 300 and 360 min and finally at 420 min.

Samples were inactivated by heating at 90°C for 10 min. Peptides were analysed by HPLC according to the protocols described in the following section.

Results of hydrolysis

Results of *in vitro* hydrolysis of the caseins are shown in Figure 7. A1 casein gave by far the greatest amount of β-CM-7 (520 µg β-CM-7/g casein at 420 min). The addition of elastase and peptidase, in particular, seemed to be particularly effective at liberating β-CM-7 in the reaction mix. The rate of formation of β-CM-7 in the reaction mix was still on a sharp incline at the final time point taken (420 and 180 min respectively).

The levels of β-CM-7 measured in hydrolysis of A2 casein was far less than that measured in the hydrolyses of A1 casein. It is difficult to tell, however, due to the presence of small quantities of A1 casein in the A2 casein, whether the β-CM-7 was formed from the hydrolysis of the A2 casein or to a small amount of A1 casein 'contaminant', or both. If β-CM-7 was formed from the hydrolysis of A2 casein, the rate of reaction was many orders of magnitude less than that observed with the hydrolysis of A1 casein.

Peptide Map from MS

Peptide maps of the end points for each hydrolysis experiment were obtained. The resulting spectra were searched for peptides with the following molecular weights:

- 889.1 (Val-β-CM-7)
- 1100.6 (A2 procasomorphin)
- 1140.9 (A1 procasomorphin)
- 579.7 (β-CM-5)
- 676.8 (β-CM-6)

In vitro hydrolysis of A1 casein showed that a peak with large intensity and m/z 889.8 was identified at 54.12 min (see Figure 8). The analogous A2 sample showed a peak of m/z 889.8 of much lower intensity at the same retention time. This peak was confirmed as Val-β-CM-7 by MS/MS (see Figure 9). This indicates that the levels of β-CM-7 in the reaction mix would not have reached a maximum, which is verified by the curves shown in Figure 7.

Relationship between Autism and β-Casein Variant Consumption

The correlation between consumption of histidine variant milks and the observation of BCM peptides in the urine of autistics was investigated.

Collection of samples

Subjects were recruited using stratified-random sampling from children who had been diagnosed with autism by qualified health practitioners. Subjects were fasted overnight and given 500ml proline or histidine milk per 70kg body weight *pro rata*. Milks were blinded and labelled "A" or "B". Urines were collected pre and post consumption of the milk, into containers, from children in six age groups up to 18 years old. Supervision of the collection was undertaken by the caregivers for the autistic children. Approximately 100 ml of urine was applied to a SEP-PAK cartridge previously equilibrated with 0.8% trifluoroacetic acid (TFA) in water.

Sample preparation and HPLC:

The absorbed peptides were eluted with 4 ml of 80% acetonitrile (MeCN), 0.8% TFA and vacuum concentrated to 0.8 ml. Concentrated samples were diluted 10-fold with MilliQ water and filtered through 0.45µ membrane filters. 100 µl samples were injected into the HPLC.

Peptides were separated by reversed phase HPLC on a Waters Alliance HPLC separations module using a Waters Symmetry C18 3.9 mm x 150 mm (5µm, 300A, part number WAT046980) preceded by a guard column, Waters Security Guard- Guard Cartridge System (part number KJO-4282) with a widepore C18 (ODS) 4 mm x 3 mm cartridge (part number AJO-4321);

Peptides were detected at 230nm. Sample was injected onto the column in 9% acetonitrile, 0.1% TFA with a flow rate 1ml/min. The individual peptides were progressively eluted from the column using the gradient described in Table 5. Selective elution was due to differences in the hydrophobicity and size of the different peptides.

TABLE 5

Gradient used to generate the peptide profile

Time (Min)	%A (0.8% TFA in water)	%B (0.8%TFA, 80% MeCN)
0	90	10
40	45	55
46	10	90
48	90	10
55	90	10

MRM mass spectrometry of peptides:

The mass spectrometer (a Perkin Elmer Sciex Triple Quadrupole API300 LC/MS/MS system) was calibrated weekly using a PPG standard as outlined in the PE Sciex API 300 manual, using MassChrom 1.0 software.

The eluate from the HPLC (flow approximately 1ml/min) was directed into the Turboionspray ion source and ions were generated and focussed using a positive ion spray voltage of 6000V. Orifice and ring voltages of 40 and 250 respectively with the temperature of the nebulising gas

set at 400 °C. BCM7, Val-BCM7 and BCM6 were identified in the urine samples using multiple reaction monitoring (MRM) of the following transitions: m/z 790.6/383.4, m/z 890/383.4 and m/z 678/270.1. Peak identities were confirmed by the use of standards.

Results

The urine from all autistic children contained low levels of BCM-6, BCM-7 and VAL-BCM-7 following overnight fasting and consumption of milk containing β-casein A2 variant (Figures 10 and 12). However, in the urine from a proportion of autistic children a marked increase (up to 10 fold) of beta-casomorphin 6 was observed following the consumption of milk containing β-casein A1 variant (Figures 10 and 11). Age-matched normal children did not have casomorphins in their urine.

Thus the consumption of histidine variants led to an increase of BCM peptides which affect normal function of the CNS, leading to neurological/mental disorders. The consumption of milk not containing histidine variants, but which did contain proline variants, did not lead to an elevation of BCM peptides.

Conclusion

These results show that there is strong analytical and epidemiological evidence to support a relationship between the consumption of β-casein variants with a histidine at position 67 in the amino acid sequence and neurological/mental disorders. This is most likely due to the release of the bioactive peptide BCM-7 and similar peptides such as VAL-BCM-7 and BCM-6 from histidine variants during digestion (Hartwig *et al.*, 1997). These peptides can in susceptible individuals bind to the μ-receptor in brain cells (Sun *et al.*, 1999) and in so doing modify behaviour (Sun and Cade 1999). No significant correlation was observed between the consumption of β-casein variants with a proline at position 67 in the amino acid sequence and deaths due to mental disorders or the presence of elevated levels of bioactive peptides in the urine of autistic subjects. This supports the hypothesis that as the bioactive peptide BCM-7 and its analogues cannot be released by digestive enzymes from proline variants (Hartwig *et al.*, 1997). The consumption of this type of milk will therefore not cause behaviour changes in susceptible individuals.

The disorder for which evidence is given in this specification is autism. Autism is to be understood to include all types of disorders in the autism spectrum. The invention is also intended to include all neurological or mental disorders which are aggravated or induced by the digestion of milk or a milk product containing the histidine variant.

INDUSTRIAL APPLICATION

Individuals who are susceptible to the adverse consequences of the histidine variant still require a source of nutrients in their diet. The industrial application of the invention is to supply such susceptible individuals with milk or milk products or nutrient products containing milk without the histidine variant. To do this herds are selected which only produce milk not containing the histidine variant. Milk from such a selected herd is then segregated from other milk and processed and supplied in the same way as any other milk.

Although the examples of this specification describe the milk not containing the histidine variant from bovines, other mammalian milk is known to contain little or no histidine variant. Well known examples of such milk are goat milk in which thus far only the proline variant has been observed (Swissprot database 2001). Sheep milk contains proline variants and no histidine variants, but does contain variants with an alanine at equivalent to position 67 in the amino acid sequence (Swissprot database 2001). As any non-proline variant will also behave like histidine variants and release BCM-7 upon digestion.

Example 1 - Selection of Milk Which does not Contain Histidine Variant

Cows are selected in accordance with the method described at lines 1 to 10 on a page 12 of WO96/14577. Milk from A2A2 cows is separated and made available either for further processing into milk products or for direct sale for the purpose of administration to individuals susceptible to mental disorders.

Example 2 - Milk typing for β-casein variants

Milk was also typed by a polyacrylamide gel electrophoresis in accordance with Example 4 on page 9 of WO96/14577 so as to ensure that it did not contain any histidine variants.

Example 3 - Formulation of Milk Product

Milk products such as food ingredients or nutraceuticals were formulated in a manner known by those skilled in the art.

References

Brudnak M A (2001)
Application of genomeceuticals to the molecular and immunological aspects of autism. *Medical Hypotheses, 57, 186-191.*

Elliot, R B, Harris, D P, Hill J P, Bibby, N J and Wasmuth, H E (1999)
Type-1 (insulin-dependent) diabetes mellitus and cow milk casein variant consumption. *Diabetologia 42, 292-296.*

Flynn A and Cashman K (1997).
Nutritional aspects of mineral in bovine and human milks. In Advanced Dairy Chemistry Volume 3: Lactose, water, salts and vitamins. Ed Fox, P F, Chapman and Hall, London. pp 257-302

Hamraeus L (1992)
Nutritional aspects of milk proteins. In Advanced Dairy Chemistry Volume 1: Proteins. Ed Fox, P F, Elsevier, London. pp 457-490.

Hartwig A, Teschemacher H, Lehmann W, Gauly M & Erhardt G (1997)
Influence of genetic polymorphisms in bovine milk on the occurrence of bioactive peptides. In *Milk Protein Polymorphism*, IDF, Brussels, Belgium, Pp 459-460.

Knivsberg A-M, Wiig K, Lind G, Nodland M, and Reichelt KL (1990) Dietary intervention in autistic syndromes. *Brain Dysfunction* 3: 315-317.

Miesel, H., 1997. Biochemical properties of bioactive peptides derived from milk proteins: Potential nutraceuticals for food and pharmaceutical applications. *Livestock production Science* 50, 125-138.

Panksepp J (1979) A neurochemical theory of Autism, *Trends in Neurosciences* 2: 174-177.

Petrilli P, Picone D, Caporale C, Addeo F, Auricchio S & Marino G (1984) Does casomorphin have a functional role? *Federation of European Biochemical Societies*, 169, 53-56.

Pihlanto-Leppälä A, Antila P, Mäntsälä P & Hellman J (1994) Opioid peptides produced by in-vitro proteolysis of bovine caseins. *International Dairy Journal*, 4, 291-301.

Ramabadran K and Banisnath M (1988)
Opioid peptides from milk as a possible cause of sudden infant death syndrome. *Med Hypothesis* 1988, Nov: 27(3): 181-7

Reichelt K L, Hole K., Hamberger A., Saelid G, Edminson P D, Braestrup C B, Lingjaerde O, Ledaal P and Orbeck H (1981) Biologically active peptide-containing fractions in schizophrenia and childhood au0tism. *Adv. Biochem. Psychopharmacol* 28, 627-643.

Reichelt KL, Knivsberg A-M, Nodland M, and Lind G (1994) Nature and consequences of hyperpeptiduria and bovine casomorphine found in autistic syndromes. *Brain Dysfunction* 7: 71-85.

Shattock P, Kennedy A, Rowell F and Berney T (1990) Role of neuropeptides in autism and their relationship with classical neurotransmitters. *Brain Dysfunction* 3, 328-345.

Shattock P and Lowdon G (1991) Proteins peptides and autism. Part 2: Implications for the education and care of people with autism. *Brain Dysfunction* 4, 323-334.

Storm H et al (1990) Beta-endorphin, human casomorphin, and bovine caseomorphin immunoreactivity in CSF in sudden infant death syndrome and controls. *Prog. Clin Biol Res* 1990: 328: 327-30

Sun Z, Cade JR, Fregly MJ and Privette RM (1999) Beta-casomorphin induces Fos - like immunoreactivity in discrete brain regions relevant to schizophrenia and autism. *Autism* 1999: Vol 3(1) 67-823

Sun Z and Cade J R (1999) A peptide found in schizophrenia and autism causes behavioural changes in rats.*Autism* 1999: Vol 3(1) 85-95

Svedberg J, De Haas J, Leimenstoll G, Paul F & Teschemacher H (1985) Demonstration of β-casomorphin immunoreactive materials in *In Vitro* digests of bovine milk and in small intestine contents after bovine milk ingestion in adult humans. *Peptides*. 6, 825-830.

SwissProt Database (2001). WWW.expasy.ch

Williams K, Shattock P and Berney T (1991) Proteins peptides and autism. Part 1: Urinary patterns in autism as revealed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and silver staining. *Brain Dysfunction* 4, 320-322.

CLAIMS

1. A method of providing nutrition to a susceptible individual while avoiding the induction or aggravation of a neurological/mental disorder in that individual, which comprises supplying to said individual milk which does not contain any histidine variant.

2. A method as claimed in claim 1 wherein said milk is in a milk product incorporating only milk which does not contain a histidine variant.

3. A method as claimed in claim 1 or 2 wherein said mental disorder is an autism spectral disorder.

4. A method as claimed in claim 3 wherein said autism spectral disorder is autism, pervasive developmental disorder or Asperger's syndrome.

5. A method as claimed in claim 2 or any one of the preceding claims when dependent from claim 2 wherein said milk product includes a non-dairy food ingredient which does not contain a histidine variant.

6. A method as claimed in claim 2 or any of the preceding claims when dependent from claim 2 wherein said milk product includes a nutraceutical.

7. A method as claimed in any one of the preceding claims wherein said milk is bovine milk.

8. A method as claimed in any one of claims 1 to 7 wherein said milk is derived from mammals other than bovine.

9. A method according to claim 8 wherein said milk is derived from goats or sheep.

10. Use of milk having no histidine variant in the manufacture of a milk product for providing nutrition to a susceptible individual while avoiding induction or aggravation of a neurological/mental disorder in said individual.

11. Use of a milk product manufactured in accordance with claim 10 in the manufacture of a nutrient composition incorporating only nutrients which contain no histidine variant.

12. Use as claimed in claim 12 wherein said autism spectral disorder is autism, pervasive developmental disorder or Asperger's syndrome.

13. Use according to either of claims 11 or 13 or claim 12 when dependent from claim 11 wherein said nutrient composition includes a nutraceutical.

14. Use as claimed in any one claims 11 to 13 wherein said milk is bovine milk.

15. Use as claimed in any one of claims 11 to 13 wherein said milk is derived from mammals other than bovine.

16. Use as claimed in claim 15 wherein said milk is derived from goats or sheep.

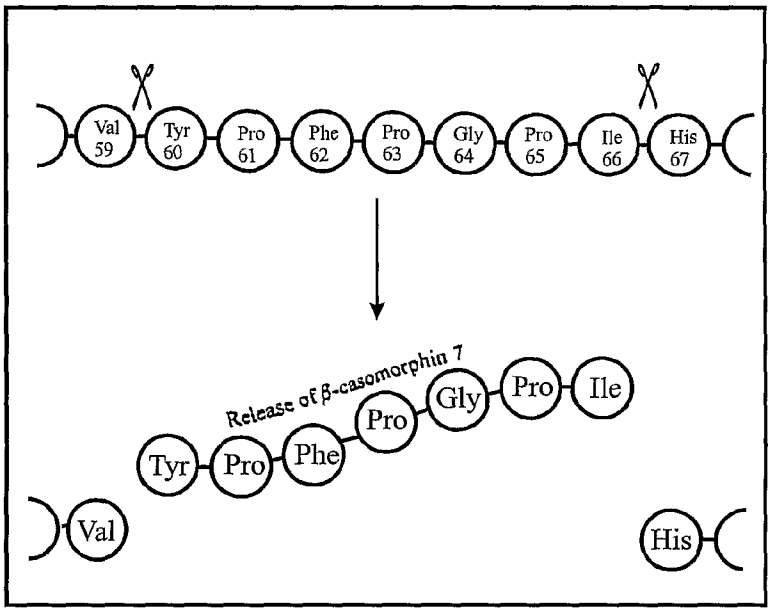


FIGURE 1

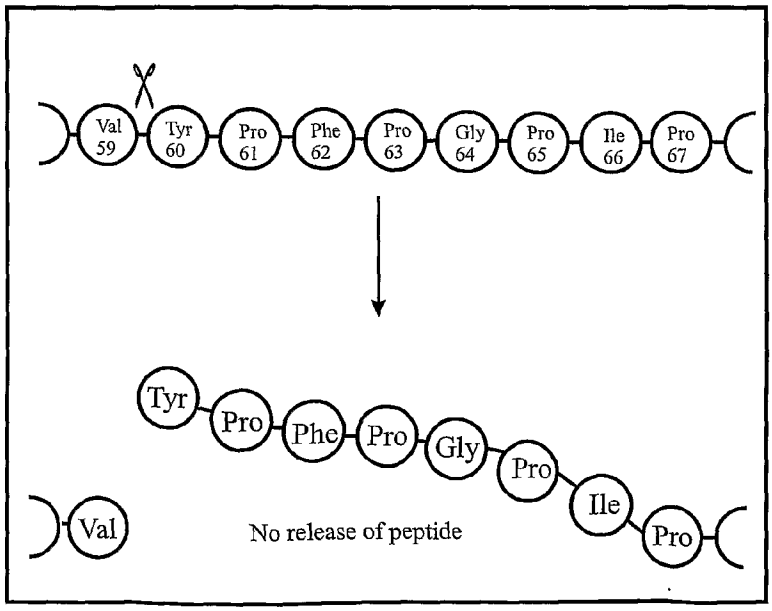


FIGURE 2

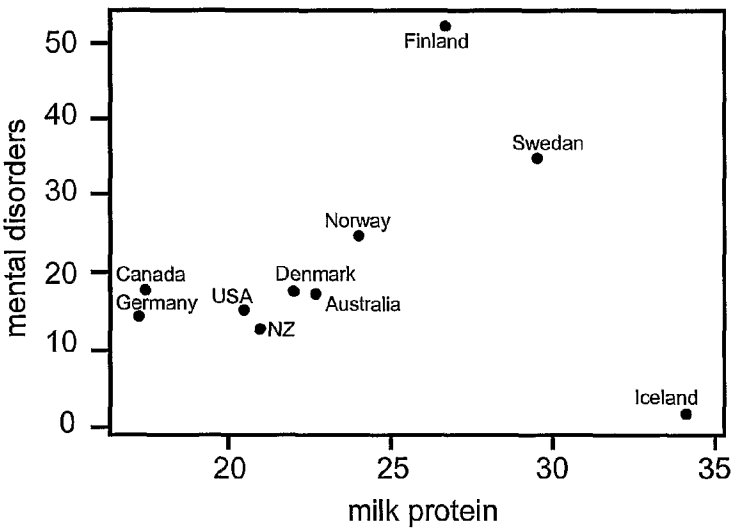


FIGURE 3

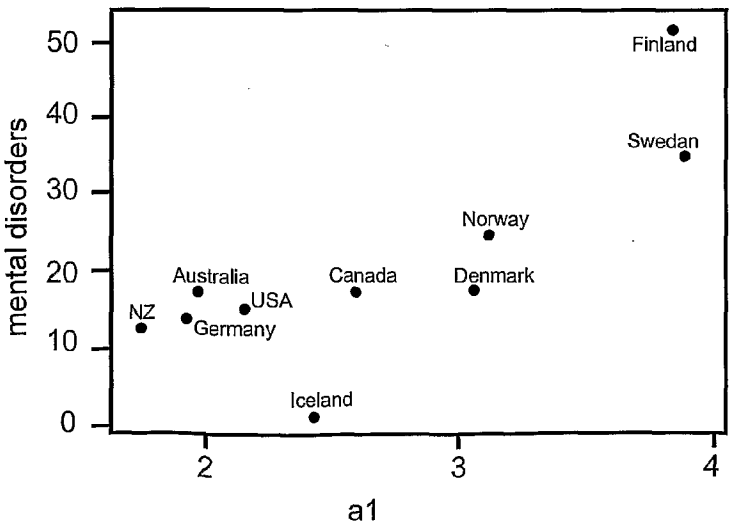


FIGURE 4

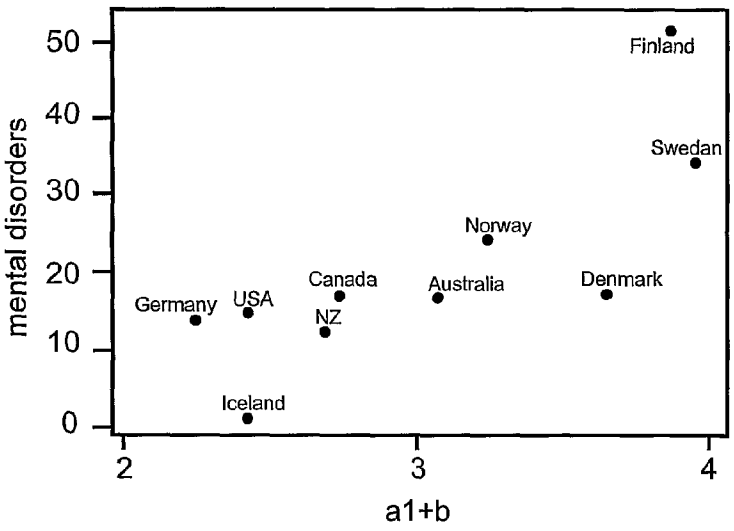


FIGURE 5

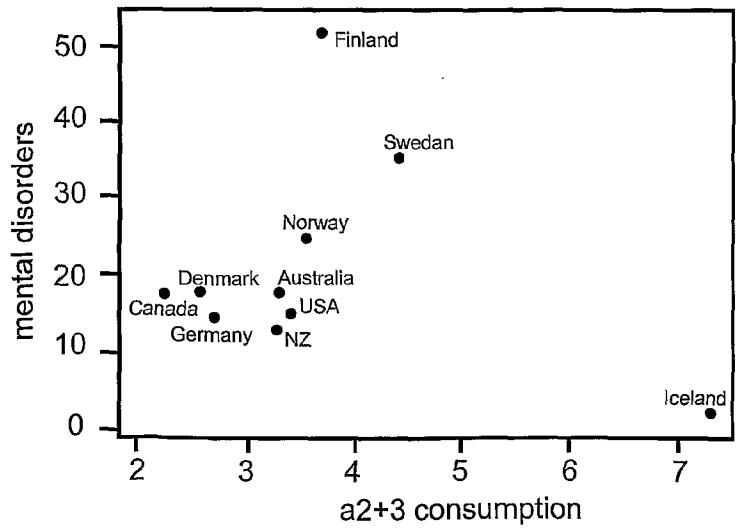


FIGURE 6

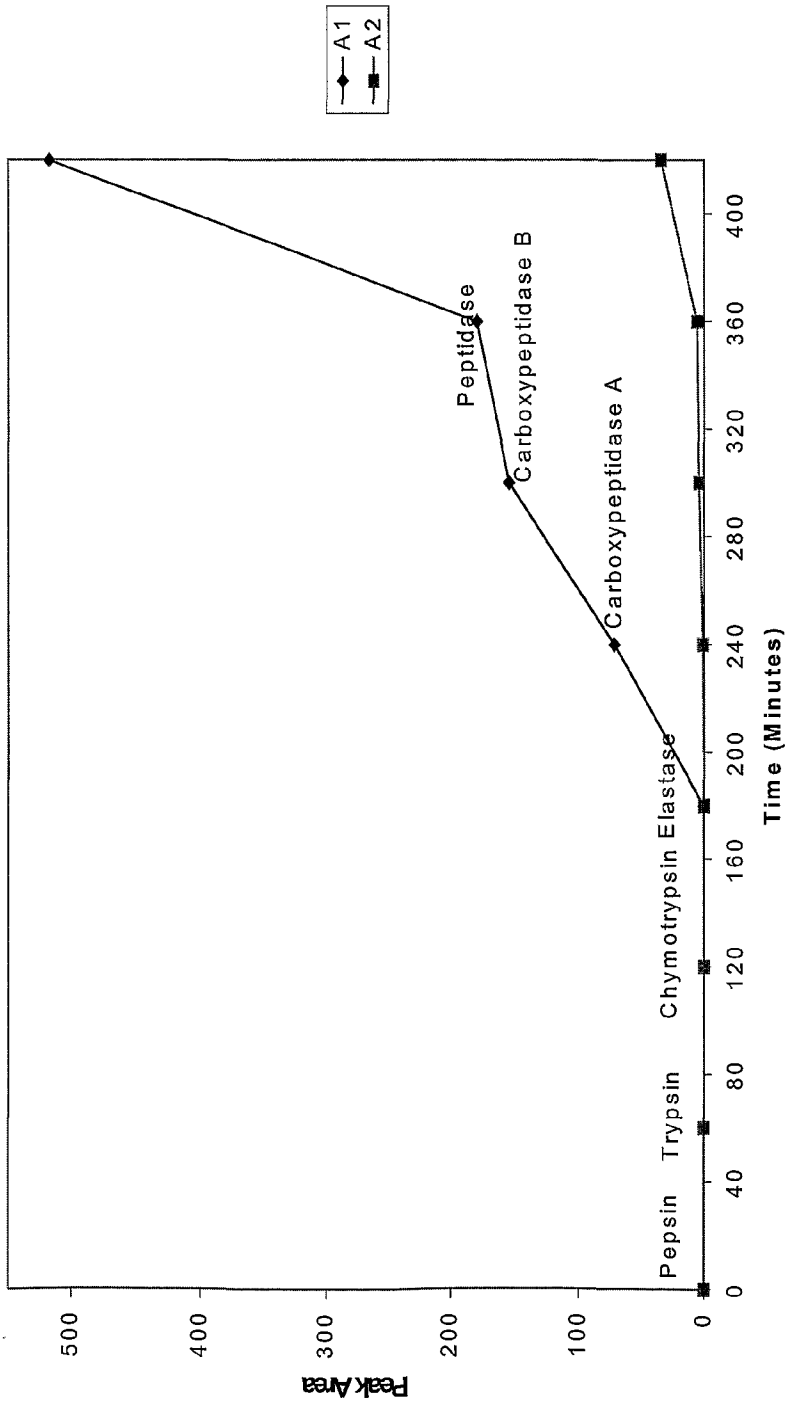

A1
A2
Peptidase
Carboxypeptidase B
Carboxypeptidase A
Chymotrypsin
Elastase
Trypsin
Pepsin
Peak Area
0
100
200
300
400
500
Time (Minutes)
0
40
80
120
160
200
240
280
320
360
400

FIGURE 7

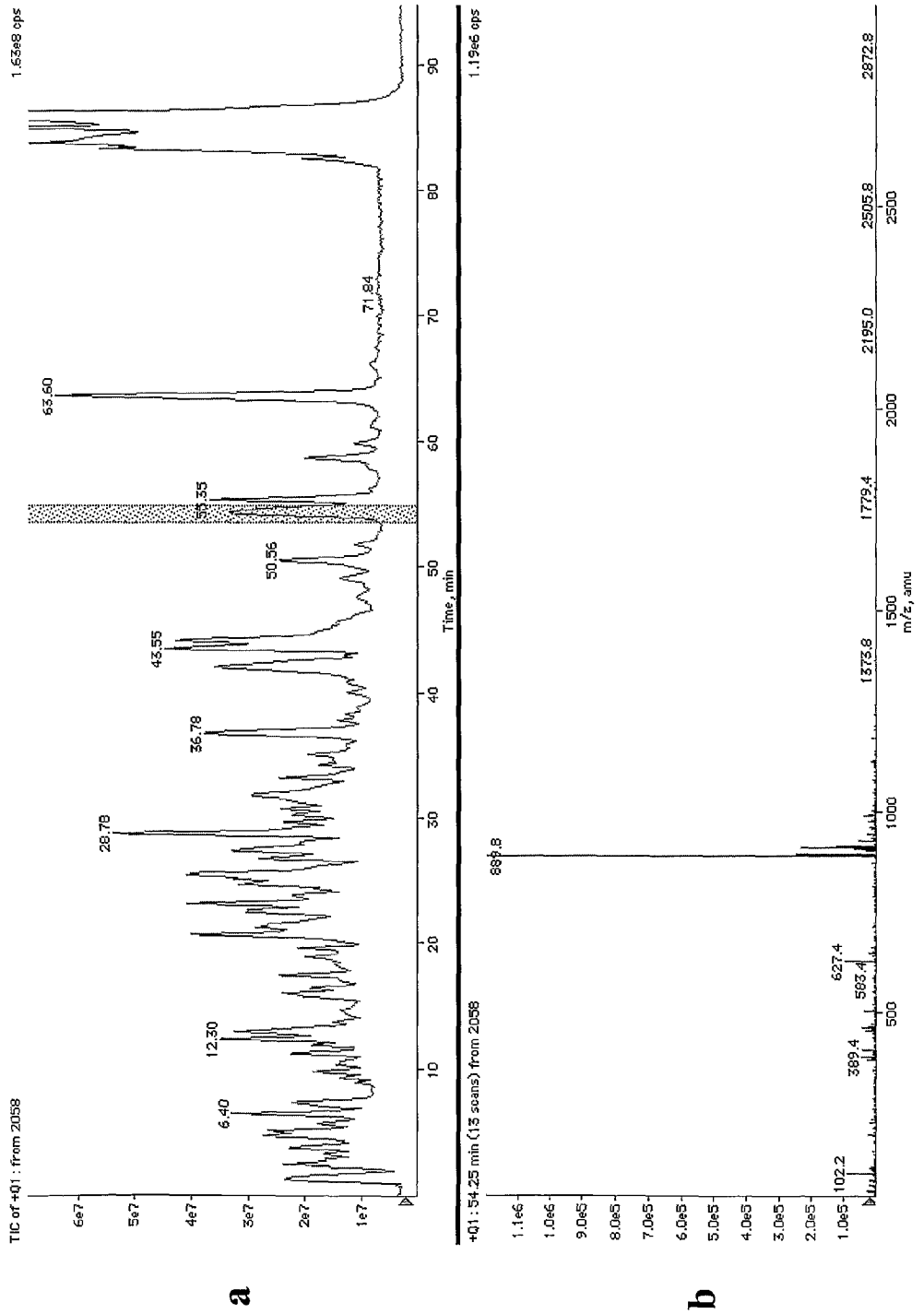

a
TIC of +Q1: from 2058
1.63e8 cps
6.40
12.30
28.78
36.78
43.55
50.56
53.35
63.60
71.84
Time, min
b
+Q1: 54.25 min (13 scans) from 2058
1.19e6 cps
102.2
389.4
583.4
627.4
889.8
1373.8
1779.4
2195.0
2505.8
2872.8
m/z, amu

FIGURE 8

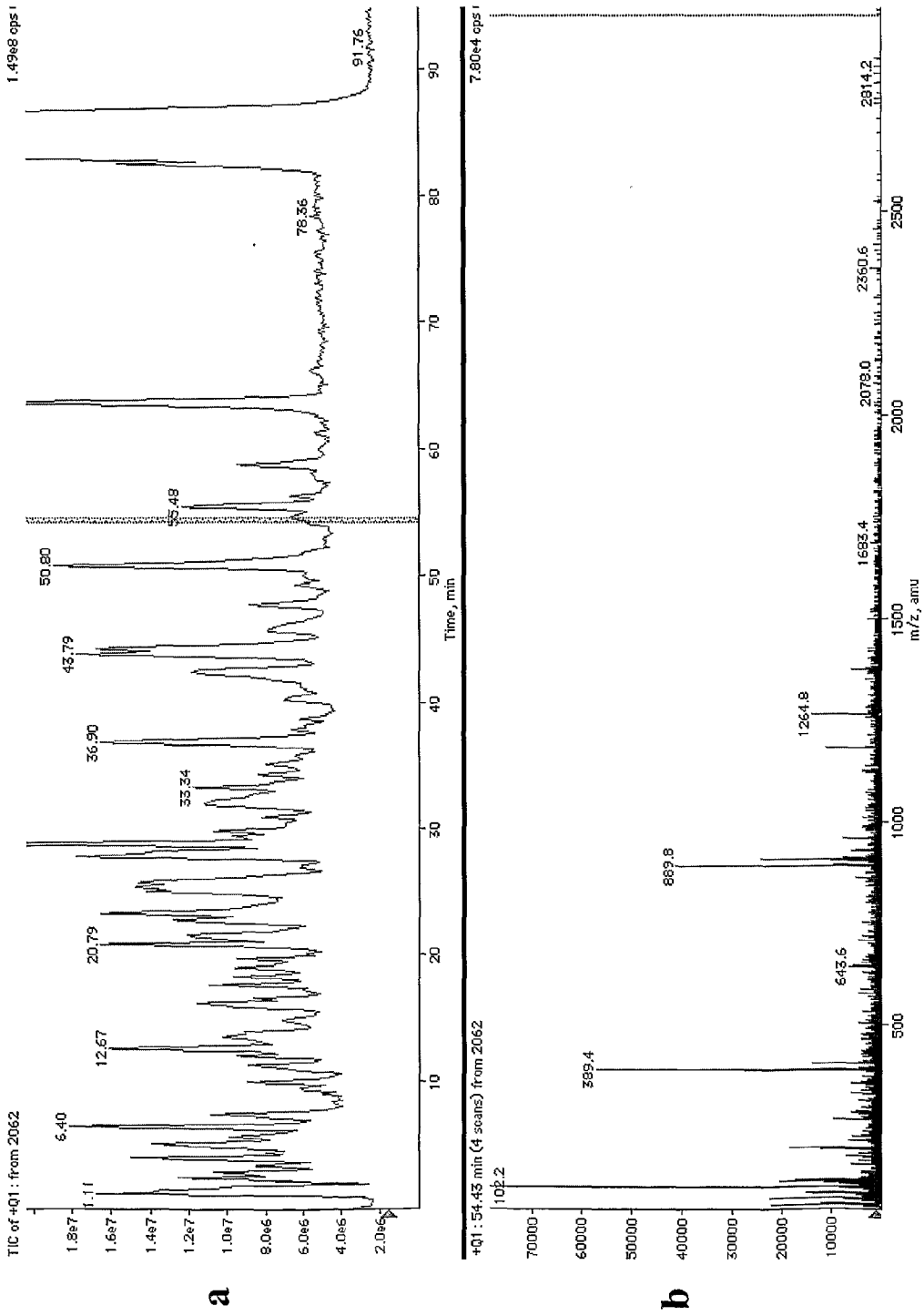

a
TIC of +Q1 : from 2062
1.49e8 cps
1.8e7
1.6e7
1.4e7
1.2e7
1.0e7
8.0e6
6.0e6
4.0e6
2.0e6
6.40
1.11
12.67
20.79
33.34
36.90
43.79
50.80
54.48
78.36
91.76
10
20
30
40
50
60
70
80
90
Time, min
b
+Q1 : 54.43 min (4 scans) from 2062
7.80e4 cps
70000
60000
50000
40000
30000
20000
10000
102.2
389.4
643.6
889.8
1264.8
1683.4
2078.0
2360.6
2814.2
500
1000
1500
2000
2500
m/z, amu

FIGURE 9

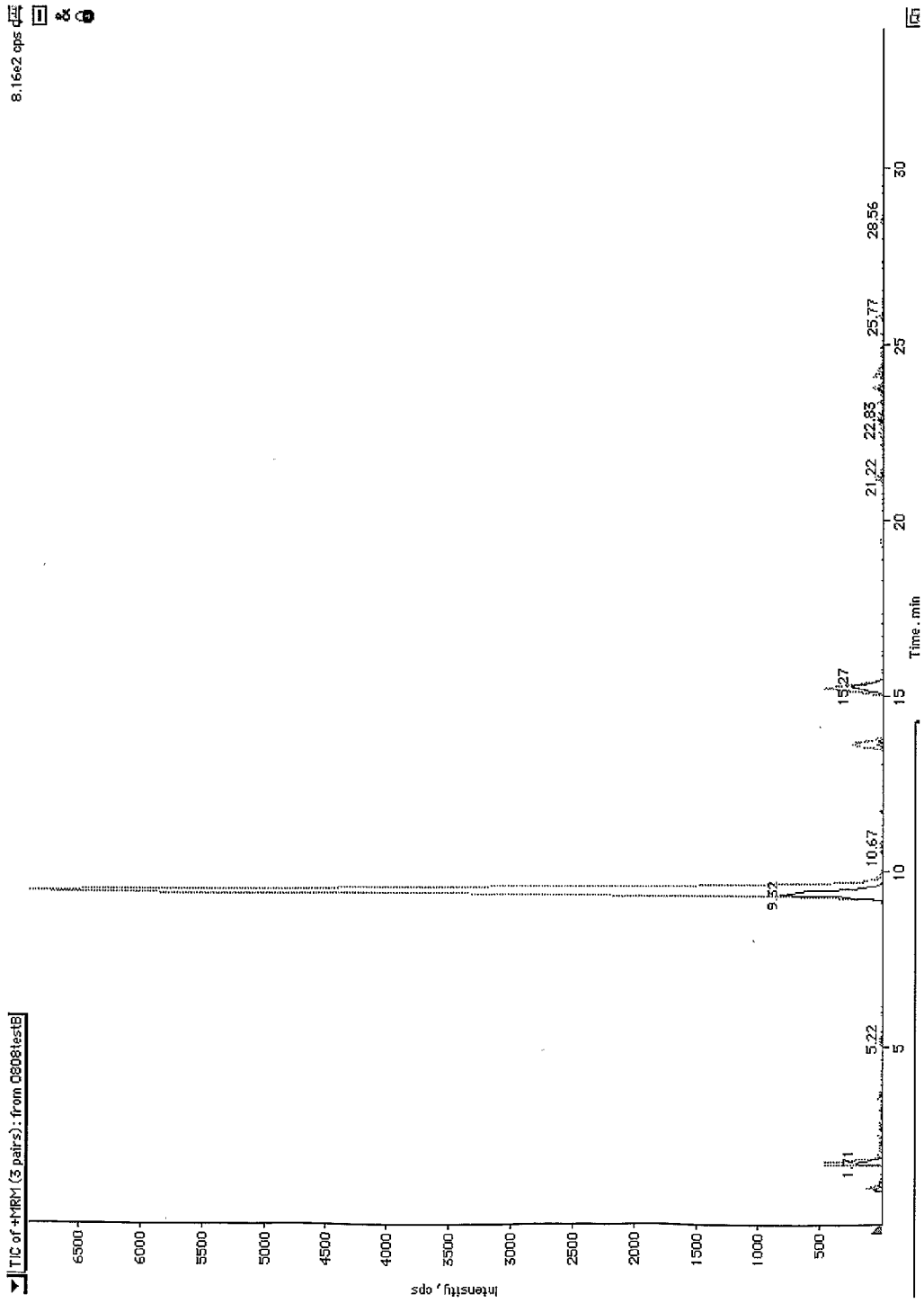

TIC of +MRM (3 pairs): from 0808testB
8.16e2 cps
Intensity, cps
Time, min
9.52
10.67
15.27
21.22
22.83
25.77
28.56

FIGURE 10

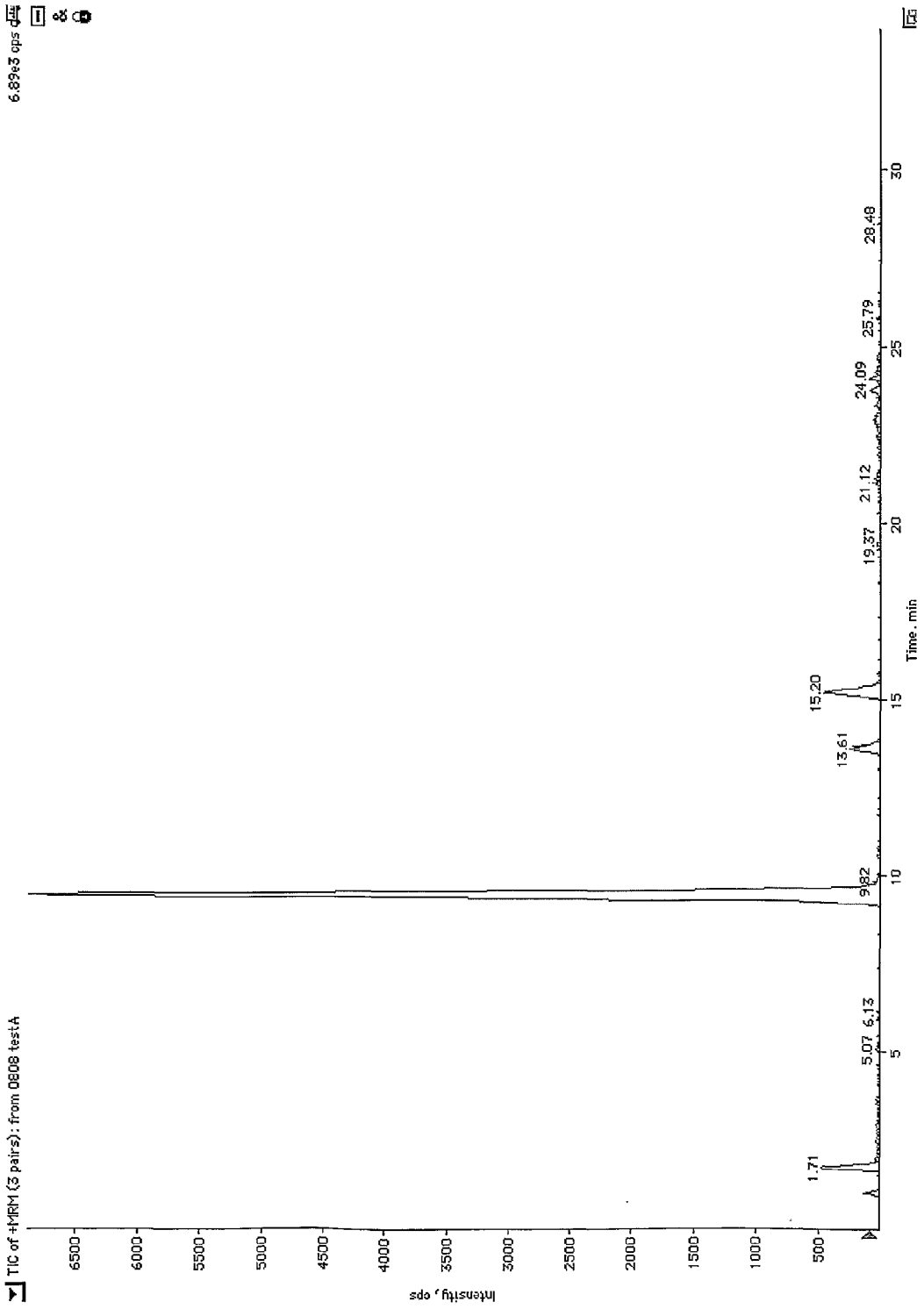

TIC of +MRM (3 pairs): from 0808 testA
6.89e3 cps
Intensity, cps
Time, min
1.71
5.07
6.13
9.82
13.61
15.20
19.37
21.12
24.09
25.79
28.48

FIGURE 11

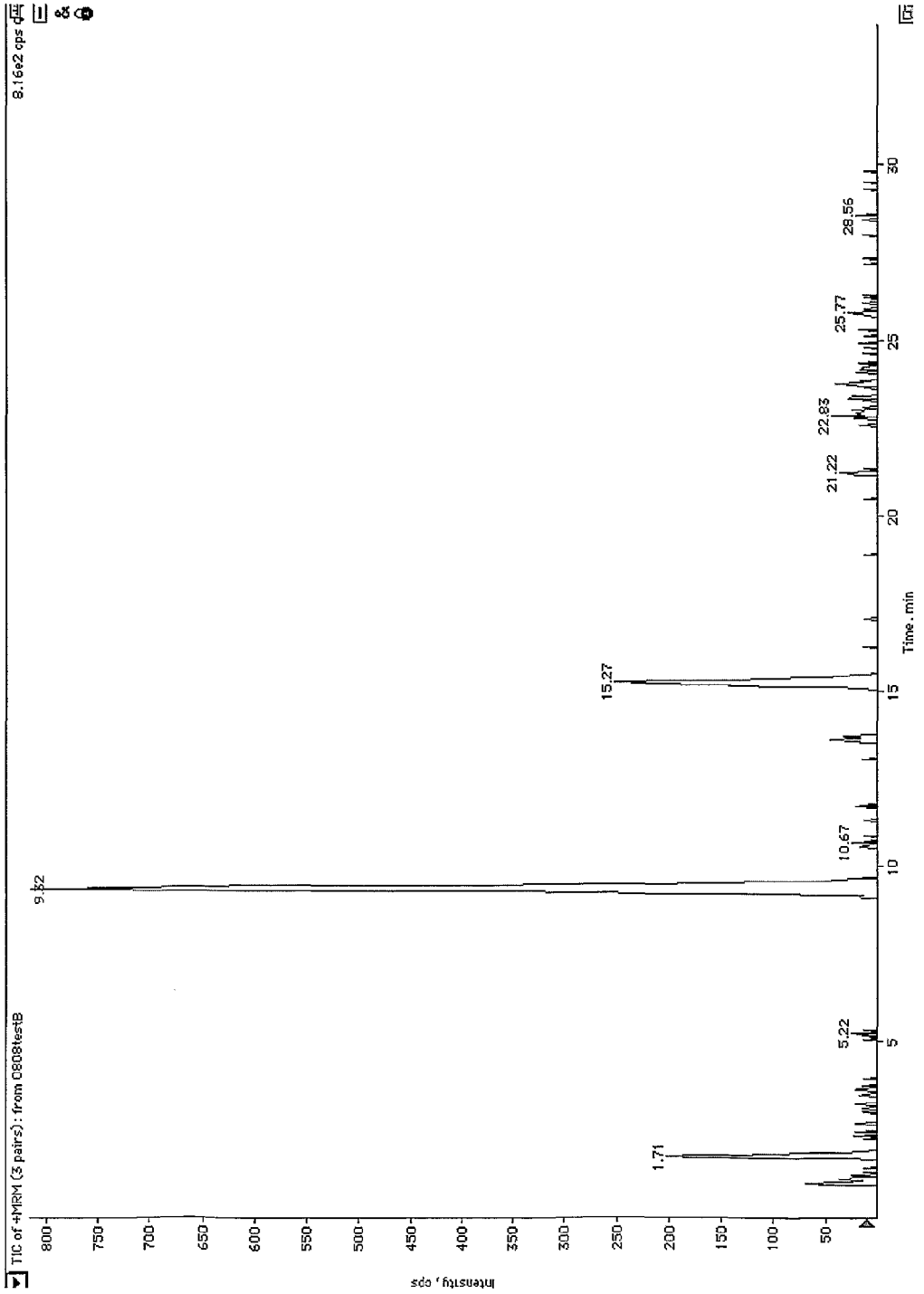

TIC of +MRM (3 pairs): from 08081estB
8.16e2 cps
Intensity, cps
Time, min
9.82
15.27
1.71
5.22
10.67
21.22
22.83
25.77
28.56

FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ01/00186

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A23C 9/00 A61K 35/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

SEE ELECTRONIC DATA BASES

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE ELECTRONIC DATA BASES

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, CA, WPIDS, FSTA: milk, autistic, histidine, autism, beta-casine, Aspergers

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. Lucarelli et al Panminerva medica (1995) 37(3) pages 137-41 "Food allergy and infantile autism" Whole Document	1 - 16
A	S. Kotsopoulos & K. M. Kutty Journal of Autism and Developmental Disorders (1979) 9(1) pages 55-60 "Histidinemia and Infantile Autism" Whole Document	1 - 16

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 October 2001	26 OCT 2001
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**CRAIG ALLATT** Telephone No : (02) 6283 2414

Form PCT/ISA/210 (second sheet) (July 1998)